Dihydroartemisinin and gefitinib synergistically inhibit NSCLC cell growth and promote apoptosis via the Akt/mTOR/STAT3 pathway

HONG JIN¹, AI-YING JIANG², HAN WANG³, YONG CAO⁴, YAN WU⁵ and XIAO-FENG JIANG¹

¹Department of Clinical Laboratory, The Fourth Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang 150001; ²Department of Pneumology, Hongqi Hospital; Departments of ³Clinical Skills Center, ⁴Pathology and ⁵Medical Research Center, Mudanjiang Medical University, Mudanjiang, Heilongjiang 157011, P.R. China

Received March 9, 2016; Accepted February 20, 2017

DOI: 10.3892/mmr.2017.6989

Abstract. Non-small cell lung cancer (NSCLC) is among the leading causes of cancer-associated mortality worldwide. In clinical practice, therapeutic strategies based on drug combinations are often used for the treatment of various types of cancer. The present study aimed to investigate the effects of the combination of dihydroartemisinin (DHA) and gefitinib on NSCLC. Cell Counting kit 8 assay was used to evaluate cell viability. Transwell assays were performed to investigate cellular migration and invasion, and cellular apoptosis was evaluated using the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay. Flow cytometry was used to investigate cell cycle distribution and the expression levels of target proteins were determined using western blot analysis. The results of the present study demonstrated that DHA (5, 10, 20, 50 and 100 µM) reduced cancer cell viability in a dose-dependent manner in the NCI-H1975 human NSCLC cell line and significantly enhanced gefitinib-induced apoptosis. Furthermore, DHA and gefitinib co-administration induced cell cycle arrest in G2/M phase, which was associated with a marked decline in the protein expression levels of G2/M regulatory proteins, including cyclin B1 and cyclin-dependent kinase 1. The addition of DHA appeared to potentiate the inhibitory actions of gefitinib on the migratory and invasive capabilities of NCI-H1975 cells. DHA and gefitinib co-administration also downregulated the expression levels of phosphorylated (p)-Akt, p-mechanistic target of rapamycin, p-signal transducer and activator of transcription 3 and B-cell lymphoma 2 (Bcl-2), and upregulated the expression of Bcl-2-associated X protein. In conclusion, the present results suggested that the combination of DHA and gefitinib may have potential as a novel and more effective therapeutic strategy for the treatment of patients with NSCLC.

Introduction

According to GLOBOCAN, lung cancer is among the most common types of cancer worldwide (1). Non-small cell lung cancer (NSCLC) comprises 85% of all types of lung cancer. NSCLC is relatively insensitive to chemotherapy compared with small cell lung cancer (2,3). Despite recent advances in the chemotherapy of NSCLC, the therapeutic efficacy of currently available agents remains unsatisfactory and the majority of patients with advanced NSCLC are refractory to medication (4,5). Therefore, the development of novel therapeutic strategies characterized by higher efficacy is imperative for the treatment of patients with NSCLC.

Gefitinib (ZD1839, Iressa) is a chemotherapeutic agent that was approved by the US Food and Drug Administration in 2003 for the treatment of patients with local or metastatic NSCLC, following failure of treatment with platinum- and docetaxel-based chemotherapeutic schemes (6). Gefitinib is an effective and well-tolerated agent; however, some patients with NSCLC are insensitive to gefitinib chemotherapy (7). Dihydroartemisinin (DHA) is the active metabolite of all artemisinin compounds and is widely used as an antimalarial therapeutic agent. In addition, previous studies have reported that DHA exhibited pronounced anticancer effects in breast, colorectal, cervical and lung cancer (8-11), whereas in combination with chemotherapeutic agents it exerted synergistic effects in the treatment of various types of cancer (12,13).

The NCI-H1975 human NSCLC cell line was established in July 1988, and is characterized by much lower sensitivity to gefitinib than other human NSCLC lines, such as A431 and H3255 (14). The present study aimed to ascertain whether the combination of gefitinib with DHA have a higher therapeutic efficacy than currently used gefitinib monotherapy. Therefore, the present study investigated the combination of gefitinib with DHA to improve the chemotherapeutic sensitivity of
NCI-H1975 cell, and explored the molecular mechanisms underlying actions of the drugs.

Materials and methods

Cell culture. The NCI-H1975 human lung adenocarcinoma cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium (catalog no. SH30809.01; HyClone; GE Healthcare Life Sciences, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS; HyClone; GE Healthcare Life Sciences). NCI-H1975 cells were maintained at 37°C in a humidified 5% CO₂ atmosphere.

Cell Counting kit 8 (CCK8) assay. Cellular viability was evaluated using CCK8 (Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer’s protocol. Briefly, NCI-H1975 cells were seeded at 5×10⁴ into 96-well plates containing RPMI 1640 medium, supplemented with 10% FBS and incubated for 24 h. When NCI-H1975 cells reached 80% confluence they were treated with gefitinib (catalog no. HY-50895; ApexBio; MedChemExpress, New Jersey, USA) or DHA (catalog no. S2290; Selleck Chemicals, Houston, TX, USA) for 24 h. Subsequently, viable cells were detected using CCK8. The absorbance of each sample at 450 nm was measured using a microplate reader (Tecan Group Ltd., Salzburg, Austria).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. NCI-H1975 cells were incubated with DHA and gefitinib for 24 h to induce cell apoptosis. Cellular apoptosis was assessed via evaluating DNA fragmentation in NCI-H1975 cells, using a Cell Death Detection kit (catalog no. 11684817910; Roche Diagnostics, Basel, Switzerland), as previously described (15). Briefly, cells were fixed for 24 h with 4% paraformaldehyde at room temperature, washed with PBS and permeabilized for 30 min with 1% Triton X-100 at 4°C. Subsequently, cells were treated with the terminal deoxynucleotidyl transferase-labeled nucleotide mix and maintained at 37°C for 1 h in the dark. Slides were rinsed and counterstained (TE 2000-U; Nikon Corporation, Tokyo, Japan) for 15 min with 10 mg/ml 4,6-diamidino-2-phenylindole (DAPI) at 37°C.

Cell cycle analysis. The effects of gefitinib and DHA on cell cycle distribution were assessed using flow cytometric analysis of the DNA content of NCI-H1975 cells, following staining with propidium iodide (PI). Briefly, NCI-H1975 cells were seeded in 6-well plates and allowed to attach overnight at 37°C. Fresh complete RPMI-1640 medium was then added, containing the desired concentrations of gefitinib (10 µM) or DHA (10 µM), and cells were incubated for 24 h at 37°C. Cells were then washed with PBS and fixed in 70% ethanol overnight at 4°C. Subsequently, cells were treated at room temperature with 80 mg/ml RNase A and 50 mg/ml PI for 30 min, and analyzed (ModFitLT version 2.0; Verity Software House Inc., Topsham, ME, USA) using a Coulter® Epics® XL™ Flow Cytometer (Beckman Coulter, Inc., Brea, CA, USA).

Cell migration and invasion analysis. To assess the effect of chemotherapeutics on the migratory and invasive capabilities of NCI-H1975 cells, cells were incubated with gefitinib (10 µM) or DHA (10 µM) at 37°C for 24 h. NCI-H1975 cells were harvested, resuspended in RPMI-1640 medium and seeded at 5×10⁴ into the upper chambers of Transwell inserts with 8 µm pore size (EMD Millipore, Billerica, MA, USA). The lower chambers contained culture medium supplemented with 10% FBS as a chemotactant. Following incubation at 37°C in 5% CO₂ for 24 h, non-migrated cells on the top of the membrane were removed with cotton swabs. Cells that had migrated to the lower membrane were fixed with 95% ethanol, stained for 1 h with 0.2% crystal violet (Sigma-Aldrich; Merck KGaA) and counted under a light microscope. Each experiment was performed in triplicate. Matrigel-coated 24-well Boyden chambers (EMD Millicore) were used in the cell invasion assay. Cells were seeded in the upper chamber in serum-free medium. The lower chambers contained culture medium supplemented with 10% FBS as a chemotactant. Following incubation at 37°C in 5% CO₂ for 24 h, non-invasion cells on the top of the membrane were removed with cotton swabs. After 24 h, cells that had invaded to the lower membrane were fixed, stained with 0.2% crystal violet (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 1 h, and counted (Image-Pro Plus 6.0, Media Cybernetics, Inc., Rockville, MD, USA) under a light microscope.

Western blot analysis. Total protein was extracted from NCI-H1975 cells. NCI-H1975 cells were digested with lysis buffer (catalog no. P0013B; Beyotime Institute of Biotechnology). Briefly, the NCI-H1975 cells were harvested and centrifuged at 12,000 x g at 4°C for 15 min and the supernatant was collected for the western blot experiment. Total protein concentration in the supernatant was determined using a Bicinchoninic Acid assay (BCA; Beyotime Institute of Biotechnology). Briefly, the BCA working solution was prepared at a ratio of 50:1 with BCA reagent A and BCA reagent B, respectively, and mixed thoroughly. Following this, 200 µl of BCA working solution was added to each of the 96-well plates containing protein samples, which were incubated at 37°C for 30 min. The absorbance was measured at a wavelength of 562 nm by a microplate reader, and the protein concentration of the sample was calculated from the standard curve. A total of 60-80 µg extracted protein samples were separated by 10-15% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk at room temperature for 1 h. Subsequently, the membranes were incubated at 4°C overnight with the following primary antibodies: Anti-mechanistic target of rapamycin (mTOR) (1:1,000; catalog no. 9664; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-phosphorylated (p)-mTOR (1:1,000), anti-cyclin B1, anti-B-cell lymphoma 2 (Bcl-2; 1:1,000; catalog no. 2872; Cell Signaling Technology, Inc.) anti-Bcl-2-associated X protein (Bax; 1:1,000; catalog no. 2774; Cell Signaling Technology, Inc.) anti-signal transducer and activator of transcription (STAT) 3 (1:1,000; catalog no. ab19352; Abcam, Cambridge, MA, USA), anti-p-STAT3 (1:1,000; catalog no. ab20647; Abcam), anti-cyclin-dependent kinase (Cdk) 1 (1:1,000; catalog no. ab18; Abcam) and anti-β-actin (1:4,000; KangChen Bio-tech, Inc., Shanghai, China). Membranes were washed three times for 10 min in PBS containing 0.5% Tween-20, and incubated with Alexa...
Fluor™-conjugated secondary antibody (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 1 h at room temperature. IRDye® 800CW Goat anti-Rabbit (catalog nos. 926-32211, C60607-15; LI-COR Biosciences, Lincoln, NE, USA), IRDye® 800CW Goat anti-Mouse (catalog nos. 926-32210, C60405-05; LI-COR Biosciences). The bands were visualized using the Odyssey Imaging system (LI-COR Biosciences) and semi-quantified using the Odyssey software version 3.0. Relative protein expression was normalized to β-actin, which was used as an internal control.

Statistical analysis. The statistical significance of the difference between groups was assessed by one-way analysis of variance, followed by Dunnett’s test for multiple comparisons. Data are expressed as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

DHA inhibits NCI-H1975 cellular proliferation. To investigate whether DHA may inhibit the growth of lung cancer cells, CCK8 assay was performed. Results demonstrated that DHA significantly inhibited NCI-H1975 cellular viability in a dose-dependent manner (Fig. 1A). The combination of DHA (10 µM) and gefitinib (10 µM) exhibited significantly enhanced inhibitory effects on NCI-H1975 cells compared with gefitinib alone (Fig. 1B); however, a lower dose of DHA (5 µM), did not appear to potentiate the effects of gefitinib (Fig. 1B).

DHA potentiates the gefitinib-induced apoptosis of NCI-H1975 cells. In accordance with previous studies (16-19), Co-treatment of DHA with gefitinib significantly increased cancer cell apoptosis. Treatment with a combination of DHA (10 µM) and gefitinib resulted in significantly higher cancer cell apoptotic rate compared with treatment with gefitinib alone. DHA administered alone demonstrated no marked effect on cell apoptosis (Fig. 2B). Representative images were indicated in Fig. 2A. These results suggested that DHA may enhance the proapoptotic effects of gefitinib, and therefore may have potential as an agent for adjuvant therapy in NSCLC.

DHA potentiates the gefitinib-induced downregulation of cyclin B1 and Cdk1 expression in NCI-H1975 cells. Cell cycle progression of NCI-H1975 cells is controlled by the sequential activation of Cdk1, whose activity depends on their association with regulatory cyclins. The formation of a Cdk1-cyclin B1 complex is crucial for the initiation of mitosis in several organisms (20,21). The analysis of cell cycle distribution using flow cytometry revealed a G2/M arrest in NCI-H1975 cells treated with gefitinib or a combination of gefitinib and DHA (Fig. 3A). Treatment of NCI-H1975 cells with gefitinib for 24 h resulted in a significant increase in the percentage of cells in G2/M phase, accompanied by a decrease in the percentage of cells in G0/G1 phase. These effects were potentiated following DHA
and gefitinib co-treatment (Fig. 3A). Furthermore, western blot analysis demonstrated that treatment of NCI-H1975 cells with gefitinib (10 µM) caused a significant downregulation in cyclin B1 and Cdk1 protein expression levels. Notably, DHA co-administration significantly potentiated the effects of gefitinib on cyclin B1 and Cdk1 downregulation (Fig. 3B and C). These results suggested that the addition of DHA to gefitinib chemotherapy may potentiate the downregulation of cyclin B1 and Cdk1 protein levels and prevent Cdk1-cyclin B1 complex formation, and thus promote G2/M phase arrest in cancer cells.

Table I. Effects of DHA and gefitinib on cell cycle distribution of NCI-H1975 cells.

<table>
<thead>
<tr>
<th>Group</th>
<th>G0/G1 phase (%)</th>
<th>S phase (%)</th>
<th>G2/M phase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>75.54±1.12</td>
<td>15.01±2.01</td>
<td>10.96±1.27</td>
</tr>
<tr>
<td>DHA</td>
<td>73.02±3.15</td>
<td>15.58±1.61</td>
<td>11.75±1.86</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>67.21±1.07a</td>
<td>18.94±2.46</td>
<td>19.83±1.42a</td>
</tr>
<tr>
<td>DHA+Gefitinib</td>
<td>60.92±1.27b</td>
<td>16.33±1.85</td>
<td>21.73±1.03b</td>
</tr>
</tbody>
</table>

*P<0.01 vs. the Control group; **P<0.05 vs. the gefitinib group. Data are expressed as the mean ± standard error of the mean.

DHA enhances gefitinib-induced inhibition of migration and invasion of NCI-H1975 cells. Representative results of the migration and invasion assays are presented in Fig. 4A. The migratory capabilities of NCI-H1975 cells were significantly reduced following treatment with DHA or gefitinib alone or in combination. Cells in the Ctrl group received no treatment. All scale bars=50 µm. (B) Inhibitory effects of gefitinib on cancer cell migration were significantly enhanced following DHA co-administration. (C) Inhibitory effects of gefitinib on cancer cell invasion were significantly enhanced following DHA co-administration. Data are expressed as the mean ± standard error of the mean of 6 independent experiments. *P<0.01 vs. the Ctrl group; **P<0.05, ***P<0.01 vs. the gefitinib group. DHA, dihydroartemisinin; Ctrl, control.

DHA enhances gefitinib-induced downregulation of p-AKT, p-mTOR and p-STAT3 in NCI-H1975 cells. Western blot analysis revealed that p-AKT protein expression levels were significantly downregulated following DHA and gefitinib co-administration. The β-actin was recognized as internal control; However, the levels of total Akt remained unaltered (Fig. 5A and B). In addition, DHA significantly enhanced the gefitinib-induced downregulation of p-mTOR, whereas total mTOR levels remained unaffected across treatment groups (Fig. 5C and D). Similarly, p- but not total STAT3 protein expression levels were significantly downregulated following combination treatment with DHA and gefitinib (Fig. 5E and F). These results suggested that DHA may enhance the inhibitory actions of gefitinib on cellular migration and invasion possibly through the regulation of p-AKT/p-mTOR/p-STAT3 pathways.

DHA enhances gefitinib-induced upregulation of Bax and downregulation of Bcl-2 in NCI-H1975 cells. Western blot...
analysis revealed that Bax protein expression levels were significantly upregulated following treatment with gefitinib. Notably, Bax protein levels were significantly increased following DHA co-administration compared with cells treated with gefitinib alone (Fig. 6A). Conversely, Bcl-2 protein expression levels were significantly downregulated following treatment with gefitinib. The effects of gefitinib were significantly enhanced following DHA co-administration. (B) Treatment of NCI-H1975 cells with gefitinib significantly downregulated Bcl-2 protein expression levels. Cells in the Ctrl group received no treatment. Data are expressed as the mean ± standard error of the mean of 3 independent experiments. *P<0.05, **P<0.01 vs. the Ctrl group; ##P<0.01 vs. the gefitinib group. DHA, dihydroartemisinin; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; Ctrl, control.

Discussion

NSCLC is among the most common types of cancer and one of the leading causes of cancer-associated mortality. Drug resistance during cancer chemotherapy is a major concern in cancer therapeutics (22). Therefore, strategies aimed at increasing the efficacy of chemotherapy may help improve disease prognosis and improve the patients' quality of life. Natural products have garnered attention as a source for the development of novel anticancer drugs. Numerous natural products have exhibited anticancer potential and may prove useful, either as single agents or in combination with existing antineoplastic drugs, in the treatment of various types of cancer (23-26).

DHA is derived from artemisinin, a natural product isolated from *Artemisia apiacea*. It has previously been reported that DHA exhibited antitumor effects in several types of cancer (27). In accordance with previous studies, the present results demonstrated that DHA exhibited anticancer effects in NCI-H1975 cells. DHA appeared to suppress NCI-H1975 cellular proliferation in a concentration manner. A prominent role has previously been reported for mTOR in cancer. Activation of mTOR signaling pathways has been demonstrated to contribute to the initiation and progression of tumorigenesis (28,29). Conversely, the inhibition of mTOR by rapamycin has been reported to enhance the chemosensitivity of cancer cells (30), and in NSCLC mTOR inhibition induced apoptosis and autophagy (31). In addition, it has been reported that DHA suppressed p-mTOR activity in ovarian cancer and rhabdomyosarcoma cells (32). Therefore, the present study evaluated the effects of DHA on mTOR. The result indicated that DHA inhibited the activity of p-mTOR. The western blot results revealed that administration of DHA alone had no effect on mTOR or p-mTOR, appearing only to potentiate the actions of gefitinib.

It has previously been demonstrated that DHA may enhance the efficacy of chemotherapy in the treatment of cancer (33). Therefore, the effects of the combination of DHA with the antineoplastic drug gefitinib were investigated in NCI-H1975 cells. Flow cytometric analysis demonstrated that the combination of DHA and gefitinib caused cell cycle arrest at the G0/G1 and G2/M phase. The percentage of cells in the G0/G1 phase was decreased by DHA+gefitinib, which was accompanied by a downregulation in Cdk1 and cyclin B1 protein expression levels. It has previously been demonstrated that Bcl-2 is a type of apoptosis-suppressing gene, which promotes cell survival by inhibiting adapters
needed for activation of the proteases (caspases) (34). Bcl-2 elevation may enhance various types of cell survival and promote cancer progression and this activity has been observed in colon, prostate and lung cancers (35-37). Bax encodes a dominant-inhibitor of the Bcl-2 protein (38) and its upregulation may promote cell apoptosis in multiple types of cancer (38–42). Furthermore, it was revealed to induce apoptosis in NCI-H1975 cells, as well as a decrease in Bcl-2 and increase in Bax protein expression levels. The expression ratio of Bcl-2/Bax was also decreased (Fig. 6A and B). Notably, although gefitinib induced cell cycle arrest and apoptosis, in combination with DHA its effects were significantly potentiated. These results suggested that DHA may act synergistically with gefitinib in cancer cells.

To explore the molecular mechanisms underlying the anticancer effects of combined treatment with DHA and gefitinib, the protein levels of Akt, mTOR and STAT3 were evaluated. The Akt/mTOR/STAT3 signaling pathway is critical in the regulation of cancer cell growth, migration and apoptosis (43,44). The present results revealed that p-Akt, p-mTOR and p-STAT3 were significantly downregulated in NCI-H1975 cells that received a combination of DHA and gefitinib; however, total protein levels of Akt, mTOR and STAT3 remained unaltered across treatment groups. These results suggested that the synergistic effects of DHA and gefitinib may be exerted through the Akt/mTOR/STAT3 pathway. However, further experiments are required to elucidate the complex mechanisms underlying the anticancer effects of DHA and gefitinib.

In conclusion, the present study demonstrated that DHA enhanced the inhibitory effects of gefitinib on cancer cell proliferation. The combination of DHA with gefitinib significantly inhibited the growth and promoted the apoptosis of NCI-H1975 NSCLC cells. The mechanisms underlying the anticancer effects presently observed may involve regulation of the Akt/mTOR/STAT3 signaling pathway.

References


